Clinical potential of oligonucleotide-based therapeutics in the respiratory system

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The discovery of an ever-expanding plethora of coding and non-coding RNAs with nodal and causal roles in the regulation of lung physiology and disease is reinvigorating interest in the clinical utility of the oligonucleotide therapeutic class. This is strongly supported through recent advances in nucleic acids chemistry, synthetic oligonucleotide delivery and viral gene therapy that have succeeded in bringing to market at least three nucleic acid-based drugs. As a consequence, multiple new candidates such as RNA interference modulators, antisense, and splice switching compounds are now progressing through clinical evaluation. Here, manipulation of RNA for the treatment of lung disease is explored, with emphasis on robust pharmacological evidence aligned to the five pillars of drug development: exposure to the appropriate tissue, binding to the desired molecular target, evidence of the expected mode of action, activity in the relevant patient population and commercially viable value proposition.

Abbreviations

AGO, Argonaute; ASO, antisense oligonucleotide; CCR3, C-C chemokine receptor 3; CPP, cell penetrating peptide; COPD, chronic obstructive pulmonary disease; CD, cluster designation; GMCSF, granulocyte monocyte – colony stimulating factor; IL, interleukin; IL-'X'R, Interleukin receptor number 'X'; LNA, locked nucleic acid; miRNA, microRNA; ncRNA, non-coding RNA microRNA; nucleotide, nt; PNA, peptide nucleic acid; PMO, phosphorodiamidate morpholinos; RISC, RNA induced silencing complex; RNAi, RNA interference; RNA-Seq; next generation sequencing of RNA; PPMO, peptide-conjugated phosphorodiamidate morpholinos; siRNA, short (or small) interfering RNA; TLR, toll-like receptor; UTR, un-translated region; βc, common beta chain.

Keywords

Oligonucleotide therapeutics, siRNA, miRNA, PNA, PPMO, delivery.

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1. Introduction

Pharmacologic intervention commonly involves the administration of synthetic small molecules, recombinant proteins or antibody-based therapeutics that target or mimic the action of proteins. However, these approaches can only be employed to target certain classes of proteins (i.e. extracellular protein in the case of antibodies). This has lead to emerging interest in targeting proteins at the mRNA level using oligonucleotide-based therapeutics such as antisense and short (or small) interference RNA (siRNA). Since the actions of oligonucleotide therapeutics are mediated through Watson-Crick binding, this approach has the advantage of being able to target all known mRNAs, as well regulatory non-coding RNAs (ncRNA) such as microRNAs (miRNA) (de Hoon, Shin, & Carninci, 2015; Djebali et al., 2012; Kawai et al., 2001). However, in developing these oligonucleotide-based therapeutics, a number of major problems have emerged including poor stability in biological fluids, potential to induce immune response and off-target actions. However, the principal problem is delivery of these large (often > 6 kDa), negatively charged (resulting from the sugar-phosphate backbone) molecules both into the target tissue and then across the plasma membrane (also negatively charged) and into the cell, where they mediate their biological action. Significantly, the delivery problems have meant that much of the effort in this area has focused upon liver disease based on the observation that this organ readily absorbs oligonucleotides following intravenous (IV) administration (Nicklin et al., 1998). Thus, Ionis (previously known as ISIS) Pharmaceuticals has recently obtained regulatory approval in the USA for mipomersan, an antisense oligoncleotide (ASO) that targets apolipoprotein B for the treatment of familial hypercholesterolemia (Santos, Raal, Donovan, & Cromwell, 2015). Nevertheless, the large surface area of the airways and their

accessibility to topical delivery suggests that the respiratory system might also represent a potential target tissue for oligonucleotide-based therapeutics.

1.1. Anatomy and physiology of the respiratory system

Lung architecture (Hasleton, 1972; Lambert, Wilson, Hyatt, & Rodarte, 1982; Lambert, 1989; R. J. Lorenz, 1966) is commonly visualized as a bunch-of-grapes (Mauroy et al., 2015) formed by 23 serial bifurcations from the trachea to the last alveolar duct (generation 22; Fig. 1A) (R. J. Lorenz, 1966). Surface area and volume in adults ranges from 24 - 69 m^2 and 2.16 - 5.23 l, respectively, varying significantly on account of age, height and disease (Hasleton, 1972; Labiris & Dolovich, 2003; Usmani, 2014). Of the ~3 l standard internal lung volume (Hasleton, 1972), 85-90% is occupied by alveoli (lung parenchyma or interstitium) (Ochs, 2014).

One of the key physiological barriers to the delivery of oligonucleotides is the thin layer of pulmonary surfactant and mucus that line lower and upper airways, respectively. The cationic and lipoproteinaceous matrix that constitutes the pulmonary surfactant of the lower airways and alveoli has a number of biological functions including mechanostructural stabilization (Parra & Pérez-Gil, 2015), surface tension reduction to enable gas exchange (Whitsett, Wert, & Weaver, 2015) and as a contributor to immune protection (Han & Mallampalli, 2015). Many of the surfactant lipid components have been successfully synthesized and are used in liposomal and lipid nanoparticle drug formulation. Surfactant proteins (SP) are principally derived from the *SPA, SPB, SPC* and *SPD* genes, expressed by type II alveolar epithelial cells. These, along with alveolar macrophages, also recycle

surfactant and together participate in innate immune responses to extrinsic inflammatory stimuli of viral (Derscheid & Ackermann, 2013; Hillaire, Haagsman, Osterhaus, Rimmelzwaan, & van Eijk, 2013), bacterial (Chroneos, Sever-Chroneos, & Shepherd, 2010; Sender & Stamme, 2014; Whitsett, 2010), fungal (Faro-Trindade et al., 2012; Ledford, Addison, Foster, & Que, 2014; Singh et al., 2015; van de Wetering et al., 2004), parasitic (Blanco, Lugones, Díaz, & Monzote) and particulate nature (Arick, Choi, Kim, & Won, 2015; Ma et al., 2015; Vattanasit et al., 2014). Alveolar macrophages also clear extraneous matter, which may or may not elicit innate and adaptive immune responses (Forbes et al., 2014).

Surfactant genes are also expressed in the larger airways (Whitsett et al., 2015) and even the nose (Gaunsbaek, Kjeldsen, Svane-Knudsen, Henriksen, & Hansen, 2014). However, the upper airways are mainly lined with mucous - a highly complex, anionic, glycoproteinaceous and gelatinous extracellular matrix (S. K. Lai, Wang, Wirtz, & Hanes, 2009) synthesized by goblet cells and submucosal glands that express *MUC5AC* and *MUC5B* and are located throughout the bronchi. The predominant structural component of mucous is large mucin polymer fibers of <10 nm size, that interact to produce a complex physicochemical organization (Shogren, Gerken, & Jentoft, 1989). Its role is the capture and elimination of large particles reaching the upper airways through inhalation. The underlying epithelium contributes to immune surveillance through both immune dampening and pathogen associated molecular pattern recognition systems (Davies, 2014). Removal of particulates is mediated by mucocilliary clearance and episodic bronchial smooth muscle contraction, i.e. cough. Materials are propelled into the trachea and then to the gastrointestinal tract for digestion. This mechanobiological phenomenon relies on the microrheology of mucous, which is more elastic in the nasopharynx and distal airways but less so in the intervening

bronchi, and is altered by disease (e.g. increased viscoelasticity due to reduced hydration) (Rubin, 2014). These changes can alter significantly mucociliary clearance rates, bacterial cell interactions with the host, particle motion within the mucous matrix and, consequently, drug delivery mechanics (S. K. Lai et al., 2009).

Respiratory disease is associated with multiple changes in the airways and lungs that can impact upon the delivery and action of oligonucleotide therapeutics. In fibrotic disease thickening of the alveoli reduces the gas diffusion barrier, in many cases preceded or accompanied by surfactant dysfunction. In chronic obstructive pulmonary disease (COPD), alveolar sac volume increases on account of loss of the type II epithelia, in turn reducing the surface area available for gas exchange (Ochs, 2014). Asthmatic patients experience dyspnea as principally airborne allergens (Hurwitz, 1955; Johansson et al., 2008; Romanet-Manent, Charpin, Magnan, Lanteaume, & Vervloet, 2002) trigger constriction of the upper airways (Noble et al., 2014) alongside inflammatory/allergic responses (Erle & Sheppard, 2014), mucous overproduction/increased viscoelasticity (S. K. Lai et al., 2009) and conducting airway/bronchial wall thickening (Olin & Wechsler, 2014). Together, these phenomena reduce the bronchial diameter during an asthmatic attack that can eventually lead to death (Dowell, Lavoie, Solway, & Krishnan, 2014). In cystic fibrosis (CF), genetic mutations (Bombieri et al., 2011) produce solute imbalances in the airways that contribute to mucous hypersecretion, altered mucous motility and dysregulated immune homeostasis, leading to opportunistic infection.

All these indications are associated with exaggerated or disrupted innate and adaptive immune responses. Indeed, recent attention has shifted onto the role of respiratory

epithelia given their importance in inflammation and the exacerbations associated with many chronic lung disease following exposure to inhaled pathogens (Zuo, Lucas, Fortuna, Chuang, & Best, 2015). Interestingly, studies have also indicated that the lung is not sterile, as commonly believed, but populated by numerous microbes (Riiser, 2015). Although sampling approaches and analytical methodologies confound this research (Salter et al., 2014), reports of pulmonary microbiome imbalances have emerged. Whether these are causal or consequential remains to be determined, however, their relevance to immune plasticity during treatment is of therapeutic interest, especially where novel therapeutics may interact with, or affect immune status.

The physiological, mechanistic and molecular differences between pathologies, coupled to the functional differences between species and organizational complexity of the tissue are such that *in vitro* 2D/3D/co-culture system and animal model use is limited, or at least challenging in their translational value (Nichols et al., 2014; Ochs, 2014; J. C. Parker & Townsley, 2008; Reus et al., 2014; Williams & Roman, 2015). This is especially the case when such approaches are used in isolation (Saturni, Contoli, Spanevello, & Papi, 2015), or without consideration of systems-level elements (Dittmar, McIver, Michalak, Garner, & Valdez, 2014; Seok et al., 2013; Takao & Miyakawa, 2015; Tsitsiou et al., 2012).

1.2 Pharmacological targeting of selective regions within the respiratory system

Airway dimensions adhere to fractal branching models (Horsfield, 1990), information essential in engineering aerosolized and dry powder systems that target drugs to the specific location within the airways and lung by inhalation (Denyer & Dyche, 2010; Laube,

2014). Thus, by controlling nebulized solution or dry powder particle size, appropriate deposition targeting can be achieved across the pulmonary tract, with minimal drug loss to the oral/nasal cavity or the environment (Fig. 1B). Drug formulations are presently evaluated for their deposition mechanics through optical particle sizing, time-of-flight spectrometry, cascade impaction and liquid impinger technologies (Denyer & Dyche, 2010; Mitchell, Bauer, Lyapustina, Tougas, & Glaab, 2011; Pu, Kline, Khawaja, Van Liew, & Berry, 2015; Zhu, Haghi, Goud, Young, & Traini, 2015).

2. Modulating RNA homeostasis with oligonucleotide-based therapeutics

A number of classes of oligonucleotide-based therapeutics have been developed although these can be principally divided into i) antisense and ii) RNA interference (RNAi)-based approaches. The first are single-stranded antisense oligonucleotides (ASO) 20-30 nucleotides (nt) in length that can either catalyse target cleavage (via RNAse H) or stoichiometrically sequester RNA targets (Fig. 2A, B). Currently, this approach has proved the most effective, with two approved ASOs that are RNase H-active (fomivirsen: approved in 1998; mipomersen: approved in 2012). Antisense-based approaches have also been developed for RNA splicing manipulation, the closest to market being drisapersen, a compound in late stage developed for the treatment of Duchenne muscular dystrophy (Fig. 2C; drisapersen, Biomarin Pharmaceutical (Disterer et al., 2014; Q.-L. Lu, Cirak, & Partridge, 2014; van Deutekom et al., 2007)). Single stranded oligonucleotides in the form of aptamers can also be used as replacements to antibodies. A marketed example is pegaptanib which targets extracellular VEGF and is licensed for use in wet macular degeneration (approved in 2004) (Sundaram, Kurniawan, Byrne, & Wower, 2013). Following the identification of siRNA-

mediated RNAi as a mechanism capable of regulating gene expression at the level of transcription and/or translation, there has also been considerable effort to develop these double stranded RNAs (dsRNA) as potential therapeutic modalities (Fig. 2D) (Moschos, 2013; Sabin, Delás, & Hannon, 2013).

The biggest challenges in the clinical progression of both antisense and RNAi-active drugs are their poor targeting to disease-relevant tissue sites and their inefficient transportation across the plasma membrane into the cytosol, where the pharmacological targets of these drugs reside. In addition, it has been necessary to address a number of other issues including stability, off-target action and immune activation.

2.1 Development of antisense oligonucleotide therapeutics

To simultaneously address the problems of antisense stability and delivery, much of the effort has focused upon the development of modified synthetic oligonucleotides (Bennett, Chiang, Chan, Shoemaker, & Mirabelli, 1992). Specifically, emphasis has been placed on development of chemistries impervious to nuclease degradation, with concomitantly enhanced pharmacokinetic properties, better cellular uptake, improved target affinity, and tailored immunogenicity profiles.

2.1.1 First Generation Antisense

Early studies on RNase H-active ASO evidenced transfection reagents as necessary to elicit appreciable function *in vitro* (Bennett et al., 1992), otherwise micromolar concentrations achieved internalization over a 50 h period through a saturable, cytoplasmic membrane

protein pathway (Loke et al., 1989). The main site of ASO activity was shown to be the nucleus (P L Iversen, Zhu, Meyer, & Zon, 1992). *In vivo* activity, however, was negligible due to the poor stability against nucleases.

The first attempts to resolve this problem involved the use of phosphorothioate backbone modifications, which imparted increased stability in biofluids and improved pharmacokinetics *in vivo* (Fig. 3A, Fig. 4). However, toxicity was observed due to interactions with heparin-binding growth factors and other compounds (Benimetskaya et al., 1997; Fennewald & Rando, 1995; Guvakova, Yakubov, Vlodavsky, Tonkinson, & Stein, 1995). It was not until many years later that serum albumin was identified as the principle (T. A. Watanabe, Geary, & Levin, 2006), but not the only carrier of phosphorothioate ASO in circulation and into the liver (Bijsterbosch et al., 2000). Uptake is presently understood to involve at least two (Koller et al., 2011), if not more endocytic mechanisms (Juliano, Ming, & Nakagawa, 2012) although this mechanism of action (MAO) is thought to account for only 20% of uptake (Geary et al., 2009). Of note, these antisense were also found to be TLR9 agonists (Krieg et al., 1995; Rutz et al., 2004) (Table 1) and demonstrated a propensity for complement activation (Advani et al., 2005; Henry et al., 1997)

2.1.2 Second Generation Antisense

The second generation of antisense involved a cadre of chemical modifications of the 2' ribose position (Fig. 3B, 4B; mixomers), or the development of entirely novel backbone structures resistant to nuclease activity, such as phosphorodiamidate morpholinos (PMO) and peptide nucleic acids (PNA; Fig. 3A). In many cases, although these modifications greatly increased the binding affinity of the ASO for its target RNA, this often coincided with the loss

of RNaseH inducing activity, as RNase H cleaves 8-12 bases from the 3' end of the ASO (Cerritelli & Crouch, 2009; Crooke, 1999; Vickers & Crooke, 2015; H. Wu, Lima, & Crooke, 1999).

Lack of RNase H induction, however, can be advantageous where the mechanism of action does not require target cleavage, such as exon modulation (splice switching or splice correction) therapy. Interestingly, some mutations in CF patients are indeed amenable to splice-switching therapy as evidenced *in vitro*. Thus, correct exon splicing has been reported in at least two separate studies modeling distinct genetic backgrounds involving aberrant splicing of *CFTR* (Friedman et al., 1999; Igreja, Clarke, Botelho, Marques, & Amaral, 2015). The therapeutic value of this approach is strongly supported by complementary studies which involve the overexpression of splicing factors as opposed to use of splice correction oligonucleotides (Nissim-Rafinia et al., 2004). There is therefore scope for expanding the evidence on the mechanistic utility of splice switching therapies in CF animal models, provided commercially appealing delivery solutions are pursued.

To address the mixomer issue around loss of RNase H activity in 2nd generation ASO attempts were made to produce so-called gapmer oligonucleotides (Fig. 4B). These were organized to feature unmodified 8-12 nt DNA sequences at the centre, flanked by modified nucleosides at the 3' and 5' ends. (Cerritelli & Crouch, 2009; Crooke, 1999; Vickers & Crooke, 2015; H. Wu et al., 1999). A number of these 2nd generation ASO have been advanced clinically with mixed outcomes (section 3).

PMOs are claimed to have higher affinity and a reduced propensity for off-target RNA binding (J. E. Summerton, 2007), with flagship programs in phase 2 and 3 clinical studies for

the treatment of Duchenne's muscular dystrophy through splicing modulation (eteplirsen (AVI-4658) and SRP-4053; Sarepta Therapeutics). Whilst preclinical data corroborated increased affinity (Q. L. Lu et al., 2005; Tanganyika-de Winter et al., 2012; B. Wu et al., 2010), including in the lung (Q. L. Lu et al., 2005; B. Wu et al., 2010), the claims of increased specificity have been challenged by others exploring off-target binding in depth (Eisen & Smith, 2008; Schulte-Merker & Stainier, 2014). Furthermore, although PMO alone showed delivery *in vitro* and *in vivo*, this can be substantially improved through conjugation with synthetic cell penetrating peptides (CPP) (Moulton et al., 2007) (peptide-conjugated phosphorodiamidate morpholinos (PPMO), or 2nd generation PMO's).

PPMOs have been recently suggested to form <90 nm, negatively charged micelles under physiologically relevant conditions (Ezzat et al., 2015), leading to cellular uptake through scavenger receptors (Ezzat et al., 2012, 2015). However, the micromolar critical micelle concentration reported by Ezzat *et al.* is higher than therapeutically relevant concentrations of drug. Although there have been eight clinical trials involving PPMOs, none have involved lung disease. However, preclinical evidence suggests efficacy after topical administration to the lung. Thus, targeting of the respiratory syncytial virus (RSV) by intranasal (IN) dosing in mice demonstrated localized antisense delivery to the bronchial airways and indicated some prophylactic value (S.-H. Lai et al., 2008; Lupfer et al., 2008) if the drug was dosed within a limited window ahead of virus challenge. Therapeutic value, however, was questionable since only a modest, <65% reduction of viral titers was reported (S.-H. Lai et al., 2008). Curiously, the extent of virologic response was comparable to the ~50% reduction of transcript levels for endogenous RNAs when these were targeted by PPMOs (Rajsbaum et al., 2014). Yet efficacious antivirals typically induce acute, multi-log (i.e. >99.9%) drops in

viral target titers. Similar results were reported in piglets (Opriessnig et al., 2011), with RNase H-inactive antisense to RSV inducing a delay, but not elimination of viral infection. Of relevance, development of viral mutational escape (Lupfer et al., 2008) has led to adoption of multi-PPMO strategies and complex dosing regimes (Patrick L Iversen et al., 2012). PPMOs might also be active against bacteria, as a single, acute (5 min), post-infection, IN 0.1 mg dose of PPMO in mice was shown to be protective against respiratory challenge with multidrug resistant *Acinetobacter* by targeting bacterial transcripts (Geller et al., 2013). However, at least some of these activities are class-level bacteriostatic effects from the covalently attached CPPs that were employed as the delivery vehicle (Wesolowski, Alonso, & Altman, 2013). In addition, PMOs also demonstrate both antibacterial and antimalarial activities (Augagneur, Wesolowski, Tae, Altman, & Ben Mamoun, 2012), a finding that further complicates the separation of gene specific from off-target effects (Eisen & Smith, 2008). PPMOs have been also proposed as inhibitors (Francis et al., 2014) of endogenous mediators of RNAi, miRNAs, and miRNA target site protectors (Staton & Giraldez, 2011). More recently, additional backbone modification of the PPMO chemistry has been sought in the so-called PMO*plus* structure (Fig. 3A). This has been evaluated clinically at phase I against lethal filovirus infectious disease (Marburg and Ebola virus), with published data in non-human primates being encouraging with regards to protective capacity (Heald et al., 2014; Patrick L Iversen et al., 2012; Warren et al., 2010, 2015). However, published data lack confirmation of the MOA of PMO*plus* chemistries. Their utility in topical administration to the lung remains unknown.

As with PMOs, PNAs also demonstrated enhanced delivery (Veldhoen, Laufer, & Restle, 2008) when conjugated to simple (Robaczewska et al., 2005; Sazani et al., 2002) and

complex (Cordier et al., 2014) CPP peptides. This includes CPPs used for PMOs (Maekawa et al., 2015) and extends to natural protein transduction domains (Fabani & Gait, 2008; Oh, Ju, & Park, 2009) and small molecule compounds such as triphenylphosphonium (Mehiri et al., 2008) or flavin (Marlin et al., 2012). Yet others have also proposed backbone modification with fluorine (Ellipilli & Ganesh, 2015) or guanidine (Dragulescu-Andrasi et al., 2006) as alternatives to peptide conjugation. With respect to the lung, activity for PNAs has been evidenced only after intraperitoneal injection with CPP-PNAs; however, daily dosing is required with PNA ASOs, whereas phosphorothioate ASOs are compatible with weekly dosing (Sazani et al., 2002).

Although PNA ASO efficacy has been evaluated in various rodent models of systemic disease (Brolin et al., 2015; Fabani & Gait, 2008; Gao et al., 2015; Rembach et al., 2004; Robaczewska et al., 2005), there are no reports examining PNA ASO utility after topical administration to the lung in the absence of any delivery modifications. This would suggest PNA ASO might not be well suited for topical dosing to the airways. Tellingly, whereas Ahn *et al.* proposed topical PNA ASO dosing against respiratory viruses (Ahn et al., 2011), at least one group has reported that a highly complex microparticle formulation is necessary to achieve any degree of efficacy. Thus, the proposed solution is a quaternary system consisting of two polymer blends, CPPs, and the bioactive PNA (Fields et al., 2015). Such approaches bear considerable development and chemistry manufacturing control costs that challenge commercial viability even within orphan disease indications. Unfortunately, evaluation of this solution in a rodent CF model (McNeer et al., 2015), exhibited modest effects. Thus, activity was reported in <0.4% of alveolar epithelia and <1% of macrophages despite particle deposition in both the small and large airways and association with 50-90%

of lung cells (Fields et al., 2015). There remain, therefore, considerable challenges to progressing in the clinic PNA chemistries for lung disease.

2.1.3 Third generation Antisense

More recently, 3rd generation antisense have been developed that make use of 2'-5' bridging groups (Fig. 3B) resulting in greatly increased stability in biological fluids, as well as higher affinity for their molecular targets. This increased affinity has resulted in the development of shorter antisense, with enhancer delivery (as a result of their smaller size) and more advantageous pharmacokinetic and pharmacodynamic properties (Dirin & Winkler, 2013; Geary, Norris, Yu, & Bennett, 2015). These antisense are also short enough to target miRNAs (anti-miRs) (Elmén, Lindow, Schütz, et al., 2008) and directly inhibit the function of the miRNA and siRNA induced silencing complex (RISC) by preventing engagement of RISC with it's RNA targets. The first 2'-5' modification to be described were 'locked' nucleic acid (LNA) (Wengel, 1999) although other structures (e.g. 2'-4' bridges) with similar performance metrics have been developed (Burel et al., 2013; Seth et al., 2009). To date, LNA remains the single 2'-5' bridging modification commercially available for research use. Interestingly, short LNA antisense enter cells *in vitro* without delivery systems i.e. in simple 'naked' saline formulations (Stein et al., 2010). The process has been termed 'gymnosis' from the Greek word for 'naked' (gymnos). This is an alternative and more efficient mechanism to 'free uptake' previously described for 2nd generation antisense (Loke et al., 1989). Crucially, activity is principally elicited in the cytosol rather than the nucleus, and possibly via nucleases other than RNase H (Castanotto et al., 2015). It remains to be

elucidated if this is a feature of all 3rd generation ASO, LNA-modified ASO alone, or an observation relevant to *in vitro* studies only. Nonetheless, 2,2,7-trimethylguanosine caps could enable nuclear targeting if necessary (Moreno et al., 2009). Presently, phosphorothioate LNA ASO have been shown to associate mainly with the liver and kidney (Straarup et al., 2010) and have been suggested to bind onto their intended pharmacological target to exert the expected MOA in up to phase IIb clinical studies (van der Ree et al., 2014). This has encouraged acquisition of the technology by Roche. However, both precursors and mature miRNA might be targeted by this approach (Gebert et al., 2013), and the methods used to evidence target engagement *in vivo* (Elmén, Lindow, Silahtaroglu, et al., 2008) could be simply artifacts of cell association carrying through tissue homogenization and high affinity nucleic acid purification, rather than true target association.

2.2. Development of RNAi-based therapeutics

Historically, RNAi therapeutics were developed on the back of discovery that delivery of exogenous dsRNA sequences of 20-27 nt length or greater, that were fully complementary to practically any part of target mRNAs, resulted in target cleavage and subsequent degradation (Fire et al., 1998). In mammals, >30 nt dsRNA were not thought to be useful in an RNAi manner as such molecules instigate antiviral responses (Bevilacqua & Cech, 1996), through Protein Kinase R engagement (Sledz, Holko, de Veer, Silverman, & Williams, 2003). However, present-day understanding of RNAi involves a considerably larger number of functions and encompasses the endogenous (miRNA) and exogenous (siRNA) mediators of RNAi operating at the transcriptional and post-transcriptional/pre-translational level (Fig. 2D) (Moschos, 2013; Sabin et al., 2013). Interestingly, endogenously expressed long (>30 nt

dsRNA-derived) siRNAs are encountered in reproductive biology, in transposon control and in cancer, operating through DNA methylation (L. Chen, Dahlstrom, Lee, & Rangasamy, 2012; Song et al., 2011; Tam et al., 2008; T. Watanabe et al., 2008; Werner et al., 2014)- a largely overlooked area. The reader is thus encouraged to consider siRNA and miRNA function in the context of RISC bioactivity as opposed to the mechanism of RISC formation as, overall, RISC functions are interchangeable for siRNA and miRNA.

The minimal bioactive complex of RNAi, the RISC complex (Fig. 2D, 5), contains one of four Argonaute proteins found in the mammalian genome, loaded with a short, ~21 nt long RNA strand of either endogenous (miRNA; Fig. 5A) or exogenous (siRNA) origin. RISC is often found in combination with multiple other regulatory proteins and uses Watson-Crick base pairing between the RISC-loaded RNA strand (referred to as the guide or antisense strand) and cellular RNAs to identify RNA targets. Target recognition relies on partial complementarity, often only between bases 2-9 from the 5' end of the guide strand (seed sequence) (Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). Where a transcript contains a sequence complementary to a miRNA seed sequence, the binding site is often referred to as the miRNA response (or recognition) element (MRE) (Leuschner, Ameres, Kueng, & Martinez, 2006; Matranga, Tomari, Shin, Bartel, & Zamore, 2005). These are most commonly found within coding transcript 3' untranslated regions (3'-UTR). However, cases of target recognition that do not involve the seed sequence at all but involve base pairing at the central (Shin et al., 2010) or 3' end of the guide strand have been documented (Sung Wook Chi, Hannon, & Darnell, 2012). The rules of target recognition are not fully described, restricting the utility of computational target prediction. Furthermore, miRNA and siRNA are

subject to 5' and 3' end modification (Azuma-Mukai et al., 2008; Burroughs et al.), which further complicates target prediction and validation.

Detailed descriptions of miRNA genomic organization and biogenesis can be found elsewhere (Moschos, 2013) and are summarized in Figure 3. In conjunction with viral and non-viral gene therapy approaches, miRNA and siRNA expression can be extensively engineered. Structurally, there are minimal differences in mature miRNA and synthetic siRNA structure (Fig. 2D), and siRNA-like synthetic miRNAs (miRNA mimics) are common tools in miRNA complementation / pharmacology studies (Perry et al., 2008). Diverse chemical modifications have been described to direct RISC precursor strand loading (Moschos, 2013) but there is no evidence to suggest AGO protein selection and RISC complex subcellular localization can be manipulated (J. H. Park & Shin, 2015).

As with single stranded ASO, the major issues with the development of RNAi mediators (siRNA and miRNA) as therapeutics has been delivery, stability, off-target actions and induction of immunological responses.

2.2.1 Demonstrating On-Target Action of siRNAs

Of the four human Ago proteins that form RISC, only AGO2 possess an RNA endonuclease, or 'slicer' function, which allows for active target cleavage (F. Li et al., 2007). Slicing of targets occurs on their phosphate backbone opposite positions 10-11 from the 5' end of the guide strand (Leuschner et al., 2006; Matranga et al., 2005), and has been shown to occur both with miRNA and siRNA guide strands (Maniataki & Mourelatos, 2005). Crucially, direct

observation of this cleavage constitutes biochemical evidence of an on-target, AGO2 RISC-mediated pathway and is typically determined using 5' rapid amplification of complementary DNA ends (5'-RACE; qualitative) followed by Sanger sequencing (Soutschek et al., 2004) or next generation sequencing (RACE-Seq; semi-quantitative (Denise et al., 2013)). Global RNA-Seq (Tabernero et al., 2013) can report the relative extent of target cleavage (knockdown), assess effects on other transcripts and any impact on splice variants. It is important to note, however, that experimental (Raabe, Tang, Brosius, & Rozhdestvensky, 2014; Sorefan et al., 2012; van Dijk, Jaszczyszyn, & Thermes, 2014) and analytical (Erhard & Zimmer, 2015; X. Liu, Zhang, & Chen, 2015; Yang & Jeong, 2013) bias in deep sequencing may severely skew observations (Lahens et al., 2014).

2.2.2 Off-target actions of siRNAs, or documenting miRNA bioactivity.

It is generally accepted that the majority of off-target actions of siRNAs are mediated through their 'miRNA-like actions' i.e. RISC docking onto the 3'-UTRs of other mRNAs, leading to the unintentional inhibition of their translation (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Jidong Liu, Valencia-Sanchez, Hannon, & Parker, 2005; R. Parker & Sheth, 2007). However, translational inhibition (as opposed to catalytic target cleavage) occurs also when siRNA forms RISC with AGOs 1, 3 and 4 which all lack the slicer function. Non-slicer RISC docking in coding regions (S W Chi, Zang, Mele, & Darnell, 2009; Nelson et al., 2007) may also lead to translational repression (Fang & Rajewsky, 2011; Schnall-Levin et al., 2011; Tay, Zhang, Thomson, Lim, & Rigoutsos, 2008) whilst docking on the 5' UTR may lead to up-regulation of translation (Ørom, Nielsen, & Lund, 2008) (Fig. 2D). These mechanisms can be assessed by determining siRNA guide strand interactions with mRNAs through cross-linking

and immunoprecipitation followed by next generation sequencing (CLIP-Seq) (S W Chi et al., 2009). Notably, this only reports target presence and/or enrichment across the entire gamut of RNAs pulled down through the assay and requires multiple processing (protease, nuclease) steps. To date, a method offering direct MOA evidence remains to be developed. Importantly, reporter constructs (Humphreys et al., 2012) disregard target transcript secondary and tertiary structures and can be misleading, as demonstrated through extensive virological research (Schopman, ter Brake, & Berkhout, 2010). Thus, single point mutations outside MRE / RISC binding sites are adequate to enable virus escape against RNAi mediators.

It is now also well established (Sabin et al., 2013) that mature RISC may translocate back into the nucleus (Bai, Liu, & Laiho, 2014; Castanotto, Lingeman, Riggs, & Rossi, 2009; Földes-Papp et al., 2009; Marcon, Babak, Chua, Hughes, & Moens, 2008; Ohrt et al., 2008; Politz, Zhang, & Pederson, 2006; Weinmann et al., 2009). Functional studies have shown that this can produce transcriptional activation (RNA activation) (L.-C. Li et al., 2006; Matsui, Chu, et al., 2013; Place, Li, Pookot, Noonan, & Dahiya, 2008; Schwartz et al., 2008; Y. Zhang et al., 2014) and repression (Ahlenstiel et al., 2012; Kim, Saetrom, Snøve, & Rossi, 2008; Younger & Corey, 2011), epigenetic remodeling (Ahlenstiel et al., 2012; Kim et al., 2008; L.-C. Li et al., 2006; Morris, Santoso, Turner, Pastori, & Hawkins, 2008; Younger & Corey, 2011; Zardo et al., 2012), miRNA precursor maturation control (Tang et al., 2012), as well as splice switching (Alló et al., 2009; Ameyar-Zazoua et al., 2012; Jing Liu, Hu, & Corey, 2012). Most of these functions involve AGO1 (Kim et al., 2008), but may also include AGO2 (Ohrt et al., 2008; Schraivogel et al., 2015) in a slicer-positive (Gagnon, Li, Chu, Janowski, & Corey, 2014) or negative (Matsui, Chu, et al., 2013) fashion. Functional partners of nuclear RISC include

transcriptional proteins (Matsui, Chu, et al., 2013; Y. Zhang et al., 2014), promoter-associated and natural antisense transcripts (Matsui, Chu, et al., 2013; Morris et al., 2008; Schwartz et al., 2008) or intergenic ncRNA (Matsui, Prakash, & Corey, 2013). Interestingly, some functions appear to integrate the spliceosome, RNA polymerase elongation and chromatin remodeling (Ameyar-Zazoua et al., 2012), and even participate in dsDNA break repair (Wei et al., 2012).

Beyond these functions, viruses have also been shown to encode RNAi mediators and manipulate RNAi homeostasis. Thus, RISC can be used to prevent 5'-3' exonuclease cleavage (Sedano & Sarnow, 2014; Thibault et al., 2015) of RNA viral genomes, promote virus replication (Fan et al., 2015), regulate viral polyprotein translation (Masaki et al., 2015) and organize the viral genome structure (Masaki et al., 2015; Mortimer & Doudna, 2013; Narbus et al., 2011). Whether some of these functions are also relevant to endogenous transcripts remains to be determined. Importantly, as with HIV *in vitro* (Schopman et al., 2010), clinical studies have evidenced mutations external to the RISC binding sites may alter RISC binding capability (Israelow et al., 2014). Yet more recently, components of AGO2 RISC have been isolated from mitochondria (Xiaorong Zhang et al., 2014), although this may not be ubiquitous across cell types (Ro et al., 2013). Mechanistic studies suggest functions in line with other cytosolic RNAi MOA (Jagannathan et al., 2015), although AGO2 RISC translocation within myocyte mitochondria and translational upregulation activity has been proposed (Xiaorong Zhang et al., 2014). Roles in mitochondrial transcription regulation have been so far documented only in plants (Dietrich, Wallet, Iqbal, Gualberto, & Lotfi, 2015), with similar studies lacking in mammals.

2.2.3 siRNA and miRNA mediated immune responses

RNAi mediators are now understood to be natural agonists of multiple pathogen associated molecular pattern receptors (Table 1) such as the toll-like receptor (TLR) family members 3 (Cho et al., 2009; Kleinman et al., 2008), 7 and 8 (Hornung et al., 2005; Judge et al., 2005). Their activation drives the release of various proinflammatory cytokines, with diverse kinetics which can vary substantially based on host species, system complexity (*in vitro* vs. *in vivo*) and delivery system type (Broering et al., 2014; Forsbach et al., 2008, 2012; Moschos et al., 2007). Crucially, the effects of at least TLR3 activation are not local: stimulation by an siRNA in the peritoneum can result in remarkable remodeling in tissues as anatomically distant as the eye (Kleinman et al., 2008). This finding contributed substantially to the abandonment of the first-in-class siRNA clinical candidate, bevasiranib. The cytokines presently implicated in TLR-mediated off target effects include alpha interferon, tumor necrosis factor alpha, interleukin (IL) 6, IL-10, beta interferon, interferon-induced protein with tetratricopeptide Repeats 1 and interferon sensitive gene 15, on account of large and small (dinucleotide) sequence motifs (Forsbach et al., 2008; Judge et al., 2005; Robbins et al., 2007; Schlee, Hornung, & Hartmann, 2006) also found in many miRNAs. Indeed, in stark contrast to common misconceptions, it is now confirmed that miRNA are TLR7/8 agonists (at least) that induce inflammatory (Fabbri et al., 2012), pro-apoptotic (W. A. He et al., 2014) and pain responses (C.-K. Park et al., 2014) through exosomal communication pathways (Patton et al., 2015); these functions depend on the miRNA sequence and the recipient cell type, and should come as no surprise to the reader versed in the immunostimulatory potential of synthetic siRNA-like molecules.

Advantageously, nucleoside analogues (Table 1 and Fig. 3B) involving 2’ ribose modifications (e.g. 2’-O-methyl, 2’-fluoro and 2’-5’ oxygen-bridged ‘locked’ nucleic acids (LNA)) incorporated in RNAi mediators act as a TLR7/8 antagonists (Robbins et al., 2007; Sarvestani et al., 2015; Sioud, Furset, & Cekaite, 2007), with at least one of these, 2’-O-methyl, altering RISC off-target effect profiles (Fedorov et al., 2006; Jackson et al., 2006). The impact of other modifications on RISC off-target effects has not been publicly disclosed. Although nucleoside suppressors of both TLR3 and TLR7/8 have been described (Karikó, Buckstein, Ni, & Weissman, 2005) (Table 1), their impact on siRNA-mediated TLR3 activation has not been assessed. Presently, reports on the impact of 2-thiouridine on slicer function are contradictory (Prakash, Naik, Sioufi, Bhat, & Swayze, 2009; Sipa et al., 2007). Overall, judicious use of one or two 2’ modified nucleosides as formulation adjuncts (e.g. 2’-O-methyl nucleosides, a natural nucleoside) or as siRNA sequence modifications can largely eliminate TLR7/8 activation potential and ablate the pyrimidine-purine specificity of RNase A (i.e. UpA, CpG, etc.) (Turner, Jones, Moschos, Lindsay, & Gait, 2007). Thus, simple changes with minimal impact of strand T_m can evade TLR7/8 induction and also drive better compound stability and increase confidence of an on-target (i.e. non-inflammatory) MOA. Presently, TLR3 evasion can be engineered only through iterative screening and testing.

Beyond the TLR family, the intracellular pathogen associated molecular pattern sensors 2’-5’-oligoadenylate synthase 1 (OAS1) (Kodym, Kodym, & Story, 2009), double-stranded RNA-activated inhibitor of translation (DAI) (Manche, Green, Schmedt, & Mathews, 1992), melanoma differentiation-associated gene 5 (MDA5) (Kato et al., 2008), and retinoic acid-inducible gene 1 (RIG-I) (Hornung et al., 2006; Marques et al., 2006; Pichlmair et al., 2006)

are also activated by RNAi mediators or their precursors. Fortunately, most are long dsRNA-specific. Among those sensitive to smaller dsRNA OAS1 is specific for the nucleotide motif NNWW(N_9)WGN (Kodym et al., 2009) whereas RIG-I is inhibited by 3' dinucleotide overhangs typical to miRNAs and endogenous siRNAs (Marques et al., 2006) (Fig. 2) or evaded through lack of 5' triphosphate use on either RISC precursor strand (Kato et al., 2008).

3. Clinical progress of oligonucleotide therapeutics for lung disease.

The original studies on lung-targeted oligonucleotide therapeutics (Moschos, Spinks, Williams, & Lindsay, 2008) expected delivery into lung cells following topical administration, despite historical data suggesting quick absorption into the circulation (Nicklin et al., 1998). This was principally due to the large size (~12 kDa) of the then fashionable siRNA compared to single stranded antisense. Thus, 90-99% reduction of gene expression (i.e. 1-2 logs) was commonly observed in cell culture whilst preclinical studies showed 2-fold (40-60%) reduction of target RNA levels (summarized in (Moschos et al., 2008)). Encouragingly, two co-administered antiviral siRNAs against separate viruses produced a 90-99% reduction in target mRNA and in tandem, alpha interferon production – a possibly advantageous feature for an antiviral RNAi drug (Bitko, Musiyenko, Shulyayeva, & Barik, 2005). Others reported that uptake across the tissue was not uniform for both ASO and siRNA encapsulated in a lipid delivery system (Griesenbach et al., 2006), and CPP conjugation to siRNA also yielded mediocre effects (Moschos et al., 2007). The inconsistencies were interpreted as experimental setup differences and variability between constitutively expressed vs. induced

RNA targets. Based upon these initial observations, a number of oligonucleotide-based therapeutics have been developed/tested in clinical studies.

3.1 RNAi mediators: ALN-RSV01 and the treatment of RSV infection.

Presently, the most clinically advanced RNAi mediator remains Alnylam's ALN-RSV01, a first generation, unmodified siRNA targeted against a conserved region of the nucleoprotein gene encoded in the RNA genome of respiratory syncytial virus (RSV) (DeVincenzo et al., 2008). Dosing the airways was expected to match the respiratory epithelium tropism of the virus (Johnson, Gonzales, Olson, Wright, & Graham, 2007), in a well-defined patient population not catered for by any on-label treatment alternatives (DeVincenzo et al., 2008), and thus likely to achieve commercial success.

3.1.1 Preclinical pharmacology.

Although mildly immunostimulating (single time point data), 2'-O-methylation of ALN-RSV01 did not affect siRNA IC_{50}, suggesting TLR activation in mouse, if any, did not contribute to antiviral responses (Alvarez et al., 2009). On-target RNAi activity *in vivo* by 5'-RACE (Alvarez et al., 2009) further justified no need for use of any nucleoside modifications. Given established *in vivo* pharmacology practice and in line with contemporary studies, drug activity was measured at the tissue level. This involved homogenization of one or more lobes of the lung and reporting virological, RNA or protein assay levels at the whole tissue level. However, direct evidence of tissue loading and retention was not attempted as no systemic absorption was expected (DeVincenzo et al., 2008). It was indeed widely assumed that the large molecular weight of siRNAs (~12.5 kDa) was recalcitrant to systemic exposure.

Rather, their anionic charge should complex with cationic alveolar surfactant (De Backer, Cerrada, Pérez-Gil, De Smedt, & Raemdonck, 2015) to piggy-back through surfactant recycling into alveolar epithelia and macrophage cytosols.

3.1.2. Clinical performance and disease biology.

Curiously, the highest dose (150 mg) tested at phase I resulted in rapid (<10 min) detection of ALN-RSV01 in blood plasma and in the first post-administration urination, at the lower detection limit of the analytical method. This was again interpreted as limited systemic exposure (DeVincenzo et al., 2008) without recourse to more sensitive methods. In an experimental clinical infection model the antiviral effect in the nasal cavity was shown as independent of TLR responses by multivariate regression analysis (DeVincenzo et al., 2010a), but no effort was made to confirm a difference between human and preclinical inflammatory responses. Thus, ALN-RSV01 was suggested to prevent the migration of the infection from the nasopharyngeal epithelium into the lower airways (Hall, 2001), an interesting proposition given RSV was classically considered a disease of the lower airways (Wright et al., 2005).

The progressive onset of symptoms from the upper to the lower airways had been documented in animal models (Gitiban et al., 2005; Richardson et al., 1978) and clinically (Hall, Douglas, & Geiman, 1975, 1976), suggesting the virus might indeed establish in the nares and progressively infect the lower airways over several days. However, no data existed confirming this hypothesis (El Saleeby, Bush, Harrison, Aitken, & Devincenzo, 2011) and the role of adaptive immunity was overlooked. Instead, comparable viral loads were

demonstrated in the upper and lower airways in the mid- to late- stages of infection (Perkins et al., 2005). Crucially, the proposed treatment strategy was not experimentally tested to determine whether antiviral protection across the upper and/or lower airways would be uniquely adequate or jointly necessary. More recent findings indicate RSV might reach the lower airways considerably sooner than clinical symptom onset might otherwise suggest (Rameix-Welti et al., 2014).

The first patient trial sought to test ALN-RSV01 utility in preventing development of RSV-mediated *bronchiolitis obliterans* syndrome, associated inflammation, infection and ultimately tissue rejection in lung transplant patients (Zamora et al., 2011a). In contrast to the previous studies (DeVincenzo et al., 2008, 2010b) the drug was administered as an inhaled, saline-formulated aerosol targeted to the lung only (Gottlieb et al., 2015a). The 0.6 mg/kg dose, ~1/3 of the 150 mg intranasal dose (DeVincenzo et al., 2008), again achieved rapid (<10 min) systemic access at the analytical assay's lower limit of detection. Furthermore, at least one patient not on corticosteroids exhibited elevated cytokine responses (Zamora et al., 2011a). No statistically significant antiviral effect was achieved, but syndrome incidence and associated sequelae reduction was reported. Thus, syndrome incidence was chosen as a sign of efficacy in follow up studies.

In phase IIb, however, the incidence rate of *bronchiolitis obliterans* syndrome was not reduced in RSV-infected individuals in a statistically significant manner, unless strict adherence to the therapeutic protocol was observed (10% incidence with treatment vs 28% incidence with placebo, $p = 0.025$) (Gottlieb et al., 2015b). Notwithstanding the impact on study power engendered through subject removal from analysis on account of protocol

adherence, complete lack of any antiviral effects in oropharyngeal washes and nasal swabs were ascribed to pulmonary targeting of the aerosol formulation. Yet no viral genome load modulation was achieved in the deep lung either: it is unclear if the analytical method used was sensitive to RISC-mediated target cleavage (G. Chen, Kronenberger, Teugels, & De Grève, 2011; Herbert, Coppieters, Lasham, Cao, & Reid, 2011; Holmes, Williams, Chapman, & Cross, 2010; Shepard, Jacobson, & Clark, 2005). It is also unclear if the reported reduction in syndrome incidence was purely on account of ALN-RSV01 treatment: of the patients in the treatment arm of the study, 71% were also receiving pulse steroid treatment vs 58% in the placebo arm.

3.2. 2nd Generation ASO therapeutics: Clinical Status.

Parallel to ALN-RSV01, topically administered 2nd generation ASO were being clinically investigated for asthma and COPD (Séguin & Ferrari, 2009). These groups also presumed lung retention, measured target modulation at the tissue level and valued disease outcome metrics more than molecular pharmacology. At the time, three clinical candidates were being progressed through to clinical programs: i) AIR645 (previously known as ISIS 369645; 2'-O-methoxyethyl chemistry; sponsored by Altair Therapeutics, an Ionis Pharmaceuticals spin-off) targeting the alpha chain common to IL receptors 4 (IL-4R) and 13 (IL-13R) (Fey et al., 2014), ii) ASM8 (TOP004 and TOP005 dual antisense formulation; 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (FANA) chemistry (Fig. 3); sponsored Topigen Pharmaceuticals, later acquired by Pharmaxis) targeting the common beta chain (βc) of the ILR receptors 3, 5 and granulocyte monocyte – colony stimulating factor (GM-CSF) and the C-C chemokine receptor 3 (CCR3), respectively (G M Gauvreau et al., 2011) and iii) ATL1102 (ISIS 107248,

another 2'-O-methoxyethyl; Antisense Therapeutics) targeting the cluster designation 49d subunit of the very late antigen 4 adhesion molecule.

3.2.1. Altair AIR645 and the targeting of IL-R4/IL-R13 in asthma.

The Altair compound was progressed on strong mechanistic rationale around the IL4/IL-13 receptor signaling system in asthma in the context of lung infiltrating, immunoglobulin E-producing, CD4+ T lymphocytes (Chatila, 2004; Karras et al., 2007). Moreover, the group had already explored 2nd generation antisense dosing to the mouse lung with other targets (Duan et al., 2005). These early studies indicated dose-dependent antisense retention in the lung but at very low levels (1.1 μg per g tissue 24 hours after a 3 mg/kg estimated dose (Duan et al., 2005)). Aerosol chamber dosing was understandably less efficient than nose-specific aerosol administration (62.4 μg per g tissue from a 3.1 mg/kg dose (Templin et al., 2000)), which also differed from historical intranasal instillation studies (Nicklin et al., 1998). Immunohistochemistry had demonstrated macrophage loading and diffuse association of antisense with lung epithelia cell types (Templin et al., 2000); however, subcellular distribution was not assessed. Nonetheless, Duan *et al.* achieved restriction of multiple asthmatic disease biomarkers efficiently modeled in mice (eosinophilia, mucus production, cytokine production, airway hyper-responsiveness), including ~60% reduction in target mRNA levels in both luminal and draining peribronchial lymph node lymphocytes. However, the mRNA data were not normalized for endogenous housekeeping gene levels (Gorzelniak, Janke, Engeli, & Sharma, 2001), undermining the claim of an on-target mechanism of action. Using a murine-specific version of AIR645, ISIS 231894, Karras *et al.* attempted to address this by measuring the reduction of IL4R alpha chain protein levels (Karras et al., 2007). This involved fluorescent cell sorting of primary lung cell subtypes in mice receiving an estimated

0.01-0.5 mg/kg nose-specific aerosol dose. At best, an ~50% reduction in IL4R alpha chain levels was observed in macrophages and lung epithelia at the highest dose, but it is unclear if multiple comparisons correction was applied in determining statistical significance, or if data distribution profiling was applied to inform linear or logarithmic scale statistical testing. Drug retention within each cell type was also not assessed. Thus, Karras *et al.* reported asthmatic response ablation but the mechanism remained unclear. Interestingly, immunomodulation appeared to be part of the effect of ISIS 231894 as revealed through cytokine profiling in a separate, antiviral study (Ripple et al., 2010). The relevance of these results appears to have been overlooked in the asthma model outputs.

Both AIR645 (primate-specific) and ISIS 231894 were tested in rodents for toxicity and tolerability, but only AIR645 was tested in monkeys (Fey et al., 2014) at 0.4 - 50 mg/kg. This involved classical pulmonary-targeted drug toxicity endpoints and complement activation but excluded cytokines (Advani et al., 2005; Henry et al., 1997). Both antisense and target levels were analyzed at tissue level (Duan et al., 2005; Karras et al., 2007). Target mRNA levels were determined in the tissue (both species) and bronchoalveolar lavage cells (monkeys only), but were expressed with reference to total RNA levels determined by substantially alternative methodologies, an approach known to result in transcript quantification artifacts. No complement activation was observed but up to 24%, dose-dependent, lung weight increases were documented in mice. Macrophage recruitment and enlargement (hystiocytosis; foamy macrophage phenotype) in the parenchyma and tracheobronchial lymph nodes was observed in both species, persisting for 13 weeks. Dose-dependent pro-inflammatory infiltrates were reported only in mice, with hystiocytosis ascribed to 'normal' alveolar macrophage function as defined by Nikula *et al.* (Nikula et al.,

2014). Importantly, the Nikula *et al.* article is an expert-level regulatory position paper on the impact of the foamy macrophage induction in response to topically dosed lung therapies. It is worth noting, that Nikula *et al.* make a clear distinction between soluble drugs (i.e. aqueous antisense formulations) and insoluble particles, such as complex formulations and small molecule aggregates (dry powders). Thus, insoluble particles do not rapidly clear from the lung and are removed specifically by macrophages (alveoli) and mucocilliary clearance (bronchial airways). This contrasts soluble antisense and siRNA which rapidly access circulation, for which there is limited clinical data available. The physiological impact of oligonucleotide-induced hystiocytosis requires further elucidation before safety is established as suggested in Karras *et al*.

Fey *et al.* calculated the clearance half-life from the lung at nine days and reported dose-dependent loading in the liver and kidney. The data were in line with parallel studies supporting lower bioavailability for topically dosed 2nd generation ASO, but contrasted earlier reports (Nicklin et al., 1998): these differences could be ascribed to the use of 1st generation ASOs by Nicklin *et al*. Yet no appreciable change in IL-4R alpha chain mRNA levels was observed. Of note, as stated in Fey *et al.*, the 1000x lower doses of AIR645 used by Karras *et al.* were apparently pharmacologically active, where Fey *et al.* reported no target modulation. The notable discrepancy was ascribed to the low-level target gene expression under naïve conditions in the Fey *et al.* study. In other words, targets expressed at low-levels would appear to be recalcitrant to oligonucleotide-mediated downregulation. Instead, Fey *et al.* postulate that antisense should be used against transcripts undergoing upregulation (e.g. activated pro-inflammatory response genes). Furthermore, antisense efficacy should be measured only when these transcripts are being upregulated, not under

baseline conditions. This contrasts evidence that transcripts expressed at low levels can be knocked down by antisense, provided the tissue is effectively transfected (e.g. apolipoprotein B downregulation in the kidney (Moschos et al., 2011)). It remains to be determined if low-level expression transcripts are malleable targets universally, or in a case-by-case basis. Clinical efficacy data have yet to reach the peer-reviewed public domain for AIR645.

3.2.2. Pharmaxis ASM8 and the targeting of CCR3 and the common βc of IL-3R, IL-5R, and GM-CSF in asthma.

The pursuit of a dual formulation consisting of two antisense drugs based on the FANA chemistry (TOP004 and TOP005; ASM8; Fig. 3) targeting two different transcripts was backed by substantial confidence in rationale (Corren, 2012). Thus, haematopoietic myeloid progenitor cells (HMC) are elevated in the circulation the lung tissue and sputum of asthmatics (and in murine models of asthma) under both naïve conditions and after allergen stimulation (Cameron et al., 2000; Dorman et al., 2004; D. S. Robinson et al., 1999; R Sehmi et al., 1996; Sergejeva, Johansson, Malmhäll, & Lötvall, 2004; Southam et al., 2005). HPCs express the receptors CCR3 and IL-5R, and differentiate into eosinophils in response to their activation (Roma Sehmi et al., 2003)(Dorman et al., 2004)(R Sehmi et al., 1997). Eosinophils are a cell type hallmark of allergic responses (Wenzel, 2006), whose concentration in the lung correlates with asthmatic disease severity (Bousquet et al., 1990). In asthmatic disease, HPCs overexpress these receptors (R Sehmi et al., 1997), parallel to the increased levels of these cytokines (Hamid et al., 1991; Humbert et al., 1997; D. Robinson et al., 1993). *Ex vivo*, soluble receptors were shown to attenuate HPC differentiation (Cameron et al., 2000).

Furthermore, clinical and primate studies with injectable monoclonal antibodies against these receptors or their corresponding cytokines, as pioneered by Leckie *et al.* (Leckie et al., 2000), indicated asthma exacerbation management could be achieved by manipulating this immune axis. Indeed, reslizumab (anti-IL-5; Teva Pharmaceutical Industries Ltd.) is presently the third monoclonal antibody on track for market approval in the USA for the treatment of asthma, and the second anti-IL-5 agent after mepolizuman (GlaxoSmithKline Plc.); benralizumab (anti-IL5-Rα; MedImmune LLC.) has also been successful in reducing asthma exacerbations at phase IIb (Castro et al., 2014). Thus, both underlying biology and clinical data contemporary to the development of ASM8 suggested that dual action against CCR3 and IL-5R could potentially achieve broader ablation of asthma exacerbation.

Encouragingly, single antisense studies *in vitro*, in rodents and non-human primates (Z Allakhverdi et al., 2006; Zoulfia Allakhverdi, Allam, & Renzi, 2002; Allam & Renzi, 2001; Fortin et al., 2006) were positive, suggesting effective target knockdown at both the RNA and protein level (CCR3 and βc) in tissue and *ex vivo* cell studies, in line with reduced airway eosinophilia and hyper-responsiveness after allergen challenge, However, the gel-based RNA quantification methodology used was poorly quantitative and no explicit MOA results such as 5'-RACE were reported throughout this work. Interestingly, additive effects were observed for the two drugs in rat models, such as reduction of lymphocyte and macrophage recruitment after challenge (Z Allakhverdi et al., 2006). Yet the results obtained in non-human primates was contradictory (Guimond et al., 2008). Thus, as with AIR645, sporadic, but dose-dependent evidence of macrophage accumulation and inflammation was reported, principally at high doses; plasma detection of the drugs was only achieved at the 2.5 mg/kg dose, with follow-on compounds detected also in the liver and kidney at the high

dose only. Curiously, very modest target knockdown was reported (16% for CCR3 and 20% for βc), only in the tracheal tissue and not in the lung parenchyma.

Clinical studies (Gail M Gauvreau et al., 2008) evidenced ASM8 recovery in lung sputum and ablation of the six-fold increase of βc mRNA ($p = 0.039$) induced by allergen challenge. However, the effect on CCR3 mRNA was not statistically significant, nor was that on the protein levels of either target. Similar observations were reported in some, but not all clinical markers of efficacy. Thus, a 46% reduction of sputum eosinophilia did achieve statistical significance, but the trend towards total leukocyte recruitment ablation did not; slightly elevated levels of macrophages were also observed. Dose-dependent, ~40% ablation of the inflammatory response was reproduced in the ensuing dose escalation study (G M Gauvreau et al., 2011), but this also coincided with a dose-dependent increase in sputum macrophages (G M Gauvreau et al., 2011). In later work (Imaoka et al., 2011), ASM8 succeeded in reducing the early (0-2 hours) and late (3-7 hours) asthmatic responses measured as a loss of forced expiratory volume in one second after allergen challenge. However, both phases of the response were ablated only by the high, 8 mg once daily, inhaled dose; the early response was not affected by a 4 mg dose. In these patients eosinophil progenitor recruitment was also significantly reduced in sputum after allergen challenge, accompanied by statistically significant reduction of $CCR3^+$ and $IL\text{-}5R^+$ HPC numbers, but not total HPC level.

Overall, despite the robust rationale, encouraging pre-clinical data and clinical success with other, injectable therapeutic modalities (antibodies) targeting the same immune signalling axis, the clinical progress of ASM8 was marred by lack of explicit MOA data, inconclusive

bioanalytical results on target RNA and protein levels and mixed clinical outcomes. It is important to note at this point that both ASM8 and it's competitor AIR645 have consistently induced macrophage recruitment into the airways, a phenotype not yet cleared as safe with regards to soluble therapeutic compounds.

3.3. Third generation ASO.

The utility of <20 nt LNA ASO was first evaluated in the same study that examined siRNA delivery to the lung at the cell type-specific level (Moschos et al., 2011). Unfortunately, as with the siRNA arm of this work, no activity was observed with LNA ASO in the lung. However, whilst siRNA and phosphodiester LNA ASO were rapidly (<15 min) eliminated in urine, phosphorothioate LNA ASO formed punctate structures within lung epithelia across upper and lower airways. These interactions appeared to be extracellular and membrane specific rather than endocytic, cytosolic or nuclear. Quantifiable amounts of phosphorothioate LNA ASO were reported only in lung macrophages, the liver and the kidney. Crucially, whereas there was no activity in lung macrophages, efficacy was observed in both the kidney and the liver: the latter was found to be indeed comparable to intravenous administration.

These data suggested inhalation might actually be a viable needle-free dosing solution for 3rd generation ASO therapies targeting systemic tissues rather than the lung, when the oral bioavailability of 2nd generation ASO varies between 2-12% even with the use of uptake enhancers (Nicklin et al., 1998; Raoof et al., 2004). Given the differences in ASO structure,

molecular weight (length), target affinity and mismatch propensity between 2nd and 3rd generation ASO, extrapolation of the LNA findings (Moschos et al., 2011) to 2nd generation ASO was actively discouraged. However, comparable doses of the phosphorothioate AIR645 (Fey et al., 2014) and LNA ASO (Moschos et al., 2011) resulted in 10x lower kidney retention of AIR645. Moreover, the low kidney expression of the RNA targeted by the LNA ASO did not prevent ASO activity as suggested for AIR645 (Fey et al., 2014). It is unclear if such limitations are target-specific or ASO-class specific, nor whether prolonged lung dosing of LNA ASO also drive macrophage recruitment.

4. Oligonucleotide Pharmacology: Time for a Reboot?

4.1 Identifying off-target actions

The plurality of biological actions mediated via RISC (Fig. 2 and Table 1), and the current observations from clinical development of siRNA and ASO therapeutics have questioned the generic use of this drug class. It is the view of the authors that these issues are also relevant to the laboratory based research using siRNAs, miRNA mimics and, perhaps less so, antisense drugs. Crucially, the vast majority of studies fail to demonstrate on-target MOA and supporting evidence is provided almost universally in an indirect fashion. From a systems pharmacology and toxicology perspective (Cook et al., 2014), confidence in safety requires elimination of unintended, off-target mechanisms throughout the drug development program, using robust and reproducible methods.

Essential in the process is the use of multiple positive and negative controls. Thus, independent verification through alternative tool compounds acting on the same molecular

target or pools such as multiple siRNA targeting the same gene (Hannus et al., 2014) can be employed. Equally, multiple negative controls are also valuable to deconvolute off-target phenomena. Unfortunately, common practice appears to be restricted to one or two (random or habitual) selections (Table 2).

Crucially, it is also important to continuous assess the potential immunomodulatory actions of oligonucleotides and not to restrict these investigations to specific time-points or inflammatory markers. One approach proposes the use of alternating modifications of the passenger strand as a universal tool for ablating TLR7/8 stimulation by siRNA (Hamm et al., 2010). This harkens to the immunostimulatory RNA strand logic of isolating TLR7/8 activation to the passenger strand where this might be mechanistically convenient (Hornung et al., 2005), e.g. in anti-viral therapeutics. It is unclear to what extent this is adequate beyond TLR7/8 activation and within which cell type/tissue context. It is thus not surprising that only two tumor suppressive miRNA analogues, miRNA-16 (TargomiRs; EnGeneIC Ltd.) and miRNA-34 (MRX34; Mirna Therapeutics Inc.), have entered clinical trials to date (Lam, Chow, Zhang, & Leung, 2015). A modified passenger strand drives careful RISC loading of the canonical miRNA-34 guide strand by MRX34 (Daige et al., 2014). Presumably, this also enables evasion of TLR7/8 activation. Conversely, modified nucleotides are used in the 5' ends of both strands of the miRNA-16 mimic TargomiRs (Reid et al., 2013), i.e. within the seed sequence (Fedorov et al., 2006; Jackson et al., 2006). It is unclear at present to what extent the modification strategies adopted with these clinical candidates affect slicer-independent RNAi pathways, successfully evade TLR recognition or impact on guide strand 3' RNA editing common to many miRNAs.

Commented [ML2]: Don't understand this?

Are TLR's 3, 7 and 8 the only concerns of the community? Natural killer cell activation has also been reported with synthetic miRNA analogues both *in vitro* and *in vivo* (S. He et al., 2013). Importantly, this report is unusual as the toll-like receptor implicated, TLR1, is not understood to be a (oligo)nucleotide pattern recognition receptor. Further studies are urgently needed.

One solution proposed to circumvent immune receptor activation has involved the use of corticosteroid inhibitors of TLR signaling (Zamora et al., 2011b). This might be questionable for some indications but can be advantageous in asthma, chronic obstructive pulmonary disease, sarcoidosis and acute lung injury where corticosteroid treatment is recommended, or even pulmonary fibrosis due to habitual use (Xaubet et al., 2013). Corticosteroids and kinase inhibitors may also be of use in deconvoluting off-target pro-inflammatory response induction in drug discovery efforts.

4.2 siRNA therapeutics: The case for next generation *in vivo* pharmacology.

The preclinical and clinical studies on pulmonary dosing of siRNA showed that topical administration of unmodified compounds could rapidly (<10 min) access circulation and be mildly immunogenic. Success was assessed using clinical outcome metrics and not molecular pharmacology: no 5'-RACE data in man were disclosed. Thus, it remains unknown if topical siRNA administration to the lung in simple formulations such as physiological saline (referred to as 'naked' siRNA) can drive on-target RNAi in man. Notwithstanding the contribution of the immune response by ALN-RSV01 activity, the correct target tissue (nasal and lower airways epithelia) was not fully defined, administration methodology was not

consistent and drug loading was not evidenced directly or followed up adequately given the systemic access outcomes.

Separate preclinical studies sought to determine which cell types within the lung were differentially loaded with oligonucleotide drugs (Moschos et al., 2011), by using cell sorting and confocal microscopy after *in vivo* dosing. Unlike previous efforts (Lomas-Neira, Chung, Wesche, Perl, & Ayala, 2005; Moschos et al., 2007; Perl et al., 2005; Xuchen Zhang et al., 2004) this group sought to quantify drug uptake and activity in specific cell types relevant to lung disease rather than reporting qualitative, low resolution (microscopy), or tissue-level data. In addition, the far-red fluorophore used in these studies, sulphonated Cy5, had an emission wavelength with very limited tissue fluorescence background, and lower lipophilicity (ΔclogD) to plain Cy5. Indeed, lipophilicity is a feature of many siRNA modifications like cholesterol (similar molecular weight and ΔclogD to Cy5), which induce better cellular uptake of oligonucleotides *in vivo* (Bijsterbosch et al., 2000; C. Lorenz, Hadwiger, John, Vornlocher, & Unverzagt, 2004; Soutschek et al., 2004; Wolfrum et al., 2007). It was thus shown that although 2'-O-methylated siRNA does indeed interact with the lung tissue, it was not subsequently adsorbed, endocytosed or delivered into the cytosol of epithelia, alveolar macrophages or endothelia. No biological activity was observed in any of the examined cell types, even though the study was powered at >94% (n=5) to measure 50% reduction in target levels. Instead, mass spectrometry showed that fully intact, sulphonated Cy5-labelled siRNA was recovered at substantial quantities within the urine of mice within 15 min of intratracheal administration (Moschos et al., 2011). Most convincingly, the colour of the urine was deep blue, i.e. the same naked eye colour of the sulphonated Cy5 dye. Indeed, a similar, unreported observation in the red (Cy3) spectrum

(Moschos et al., 2007) had been previously dismissed as incidental hematuria and remained uninvestigated. Simple allometric scaling on heart rates between species, would translate these findings to siRNA detection in urine ~140 min after administration in man. Although direct comparisons of the compounds tested in mice (Moschos et al., 2007, 2011) to ALN-RSV01 (DeVincenzo et al., 2008; Zamora et al., 2011a) is difficult, rapid (<10 min) circulation access in clinical subjects supports a common absorption and elimination mechanism in both species. As 5'-RACE was not attempted in these studies, it remains unclear if non-quantitative 5'-RACE can report residual RNAi and contribute towards misinterpretation of off-target phenomena as on-target 'slicer' RNAi.

5. Concluding remarks and future challenges

At the time of writing, Alnylam programs involving siRNAs-based approaches to the treatment of genetic and cardiometabolic disease have eclipsed ALN-RSV01 and associated projects, with RSV product development apparently focused in Japan. Elsewhere, attention on RNAi mediator therapeutics for the lung has shifted onto solving the problem of cytosolic delivery (Clark et al., 2013), whereas ongoing clinical trials in the USA are concerned with systemic, non-lung cancer, skin and ocular disorders, almost invariably involving advanced drug delivery systems (Lam et al., 2015). Nevertheless, respiratory medicine is rich in marketed and clinically advanced candidates, some of which are small molecules cleverly designed for topical administration (Jones et al., 2011) and thus are exceptionally competitive commercial propositions against modalities with more complex chemistry, manufacturing and controls requirements.

The studies on the antisense based AIR645, ASM8 and associated compounds indicated effective tissue/cell loading, potentially on-target (mRNA) molecular activity for ASM8 (but not necessarily for AIR645) and effective ablation of asthmatic phenotypes. However, no explicit 5'-RACE MOA data, evidence of sub-cellular localization was evidenced or ASO association with RNase H/intended molecular targets was reported, in line with the so-called three pillars of drug survival (Morgan et al., 2012) and subsequent emerging principles in drug development (Cook et al., 2014). The primary outcome of a phase IIa study for AIR645 (NCT00941577) was not met, and Altair Therapeutics was eventually shut down. In contrast, the efficacy of ASM8 at phase II has not lead to the termination of the programme. A follow up phase II study was withdrawn before enrolment in 2012 (NCT01380236), however ASM8 is listed as an active programme by Pharmaxis. Importantly, none of the adverse events observed with ASM8 were definitively drug-related despite the dose-dependent effect on alveolar macrophages, and no complement activation was observed (G M Gauvreau et al., 2011). Thus, without recourse to drug delivery systems, the FANA antisense chemistry remains presently the most promising candidate in realising the clinical potential of oligonucleotide therapeutics for the lung. However, in the absence of explicit 5'-RACE data it is presently uncertain that the action of ASM8 can be ascribed, fully or in part, on an on-target MOA.

In moving forward, it will also be important to take into consideration that both 2nd and 3rd generation ASO are principally loaded in alveolar macrophages, and administration of 2nd generation ASO (at least) promotes macrophage accumulation in the airways. Indeed, concerns around lung macrophage effects have been upgraded to promote use of exploratory inflammatory / toxicology biomarkers (Alton et al., 2012) as encouraged by

Cook *et al*. To date, the activation status of resident and recruited macrophages in the lung and any chronic tissue effects remain unclear (Forbes et al., 2014). Crucially, macrophage recruitment may result in respiratory disease exacerbation (Zasłona et al., 2014), an observation of particular relevance to proposed anti-inflammatory antisense therapeutics with documented macrophage recruitment propensity such as both the Ionis and Pharmaxis chemistries. Moreover, 3rd generation ASO have been very recently shown to harbour an off-target class effect that mediates long (>125kb) transcript degradation in an RNase H-dependent manner (Burel et al., 2015) con-currently to on-target MOA. Thus, clinical success for the oligonucleotide therapeutics class in lung disease will pivot on thorough exploratory toxicology studies (Cook et al., 2014; Forbes et al., 2014) *in vitro*, *in vivo* and following chronic exposure trials that effectively compartmentalise on-target value proposition against off-target effect tolerability.

Thus far, the history of oligonucleotide therapeutics shows that success hinges on proactive pursuit of evidence closely aligned with the so-called 5 pillars of drug discovery (Cook et al., 2014). The shortest route to the clinic more often than not hides pharmacological challenges that can lead to costly, late stage failure. This risk can be managed either through documented design approaches (e.g. TLR7/8 avoidance) or systematic screening towards clear, hypothesis-driven and exploratory elimination of toxicological and off-target risks. Key barriers to success have been perpetuated assumptions around adequate drug exposure (pillar 1) and molecular target engagement (pillar 2) extrapolated from tissue loading data, as well as neglect over MOA and off-target effect quantification (pillar 3). This is aligned to the unfortunate continuation of 'dogma' adoption, such as the artificial segregation of the molecular functions of slicer-mediated RNAi and miRNA-mediated RNAi (e.g. (Lorenzer,

Dirin, Winkler, Baumann, & Winkler, 2015), despite calls against this (Moschos, 2013; Sabin et al., 2013). Fortunately, companies focusing on oligonucleotide therapeutics are almost invariably accurate in selecting the appropriate patient population (pillar 4). However, in the absence of orphan indications, as is the case in many lung diseases, the apparent need for delivery solutions makes the commercial potential (pillar 5) of this drug class an exceptionally challenging proposition. The true impact of the foamy alveolar macrophage, and the true need for solutions circumventing its' elicitation is likely to catalyse ultimate success.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Figures

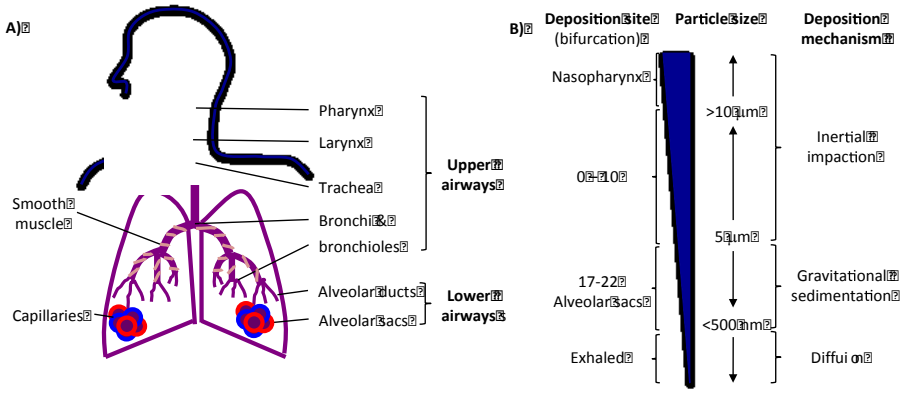

Figure 1: Anatomy of the lung, airway dynamics and drug delivery. A) The nasopharynx, trachea and first bronchial bifurcation (upper lung / large airways (>2 mm diameter)) lead to conducting airways (< 2 mm diameter: bronchi and bronchioles) that split into alveolar ducts (lower / deep lung or small / peripheral airways) (Labiris & Dolovich, 2003). Alveolar ducts conclude into alveolar sacs wherein gaseous exchange takes place. Bronchi and bronchioles are ringed tangentially by smooth muscle (R. J. Lorenz, 1966), which controls airflow through constriction and relaxation (Gosens & Grainge, 2015), whereas alveolar sacs are surrounded by capillaries, forming a short diffusion gradient for gas exchange. B) Particle dimensions and deposition mechanics by airway compartment (Heyder, 2012; Labiris & Dolovich, 2003); maximal efficiency is achieved with 1-3 µm particles, whereas <500 nm particles may diffuse into the alveolar tissue where airflow is low, but are generally exhaled.

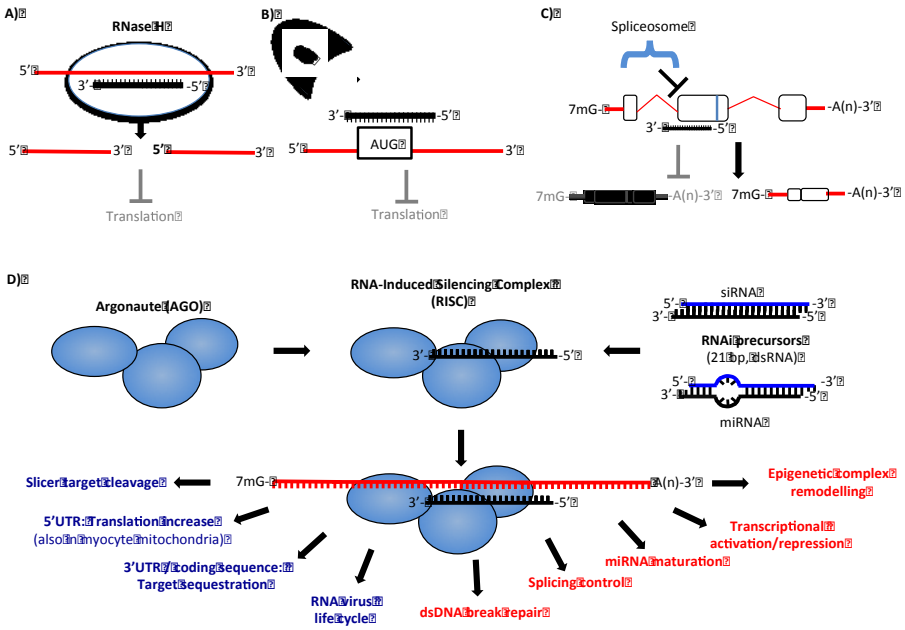

Figure 2: Classes of oligonucleotide drugs used in the lung and their mode of action. Oligonucleotide therapeutics (bold black lines) recognize molecular targets (red lines) through Watson-Crick base pairing. (A) ASO form DNA::RNA heteroduplexes in a sense (target) – antisense (drug) manner, recruit RNase H and endonucleotically cleave target RNA to create a novel 5' end (bold), resulting to target degradation and translation inhibition. (B) Alternatively, translation inhibition can be directed by targeting ASOs to translation start sites in transcripts (AUG box), preventing access to eukaryotic translation initiation factors (eIF). Both of these MOAs are active on coding and ncRNA, with stoichiometric inhibition in the latter involving steric hindrances to nucleic acid or ribonucleoprotein complex formation (C) Premature stop codons, cryptic exons and nonsense mutations (etc.) can be 'skipped' by directing spliceosome activity. The clinical implementation in Duchenne's Muscular Dystrophy uses ASOs to stop the spliceosome from including an exon (red boxes) containing a premature stop mutation (vertical grey line), so that the translated protein may retain

some function (Becker phenotype) (Disterer et al., 2014; Q.-L. Lu et al., 2014; van Deutekom et al., 2007). (D) The minimal bioactive complex of human RNAi, the RNA-induced silencing complex (RISC), consists of a ~21 nt strand of RNA in length (black; 'active' or 'guide' strand (Gu et al., 2011; Khvorova, Reynolds, & Jayasena, 2003; Krol et al., 2004)) loaded into one of the four Argonaute (AGO) proteins (Kawamata & Tomari, 2010) from an endogenous (miRNA) or exogenous (siRNA) dsRNA precursor. The reverse complement 'passenger' strand (blue) is discarded. The outcome of target interaction with RISC depends on the AGO component, site of RISC interaction within the target (e.g. coding or untranslated regions (UTR)), degree of complementarity and subcellular complex localization, i.e the cytosol (blue functions) or the nucleus (RED functions).

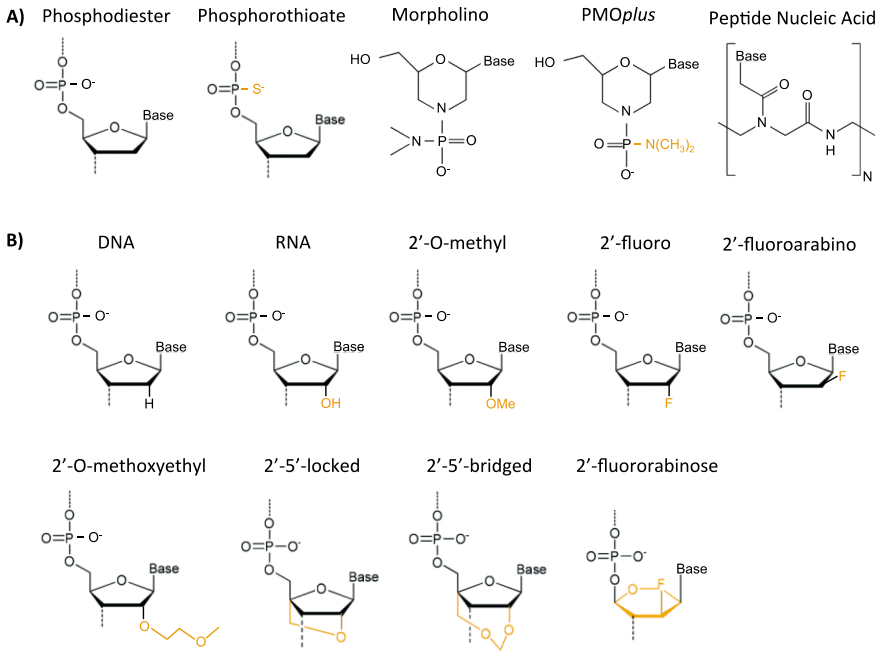

Figure 3: Key backbone and nucleoside modifications used in oligonucleotide therapeutics. (A) The most popular oligonucleotide modification (orange) of oligonucleotide backbones replaces one oxygen with a sulfur group. Morpholinos (PMO) have an uncharged backbone and six-membered rings replacing riboses that do not impact on solubility, appear to increase affinity (melting temperatures with RNA targets, ~20oC increase per ASO (Summerton & Weller, 1997)). The MOA of PMO is restricted to RNase H-free mechanisms (J. Summerton, 1999) and involves translation inhibition, splice switching and miRNA blockade (Eisen & Smith, 2008). Peptide nucleic acids (PNA) use entirely acyclic backbones conveying generally neutral charge and robust nuclease resistance and improve target affinity (Nielsen, Egholm, Berg, & Buchardt, 1991), evidenced even in siRNA modification (Gong & Desaulniers, 2012). They direct non-RNase H MOA (Uhlmann, 1998) in eukaryotes and prokaryotes (Pooga, Land, Bartfai, & Langel, 2001). (B) Nucleoside modifications have

principally involved substitution of the 2’ H (DNA) or OH (RNA) group with more complicated alkyl groups, but also 2’-5’ linking groups and 2’ arabinose stereoisomers such as the 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2’-fluoroarabino, or FANA) chemistry.

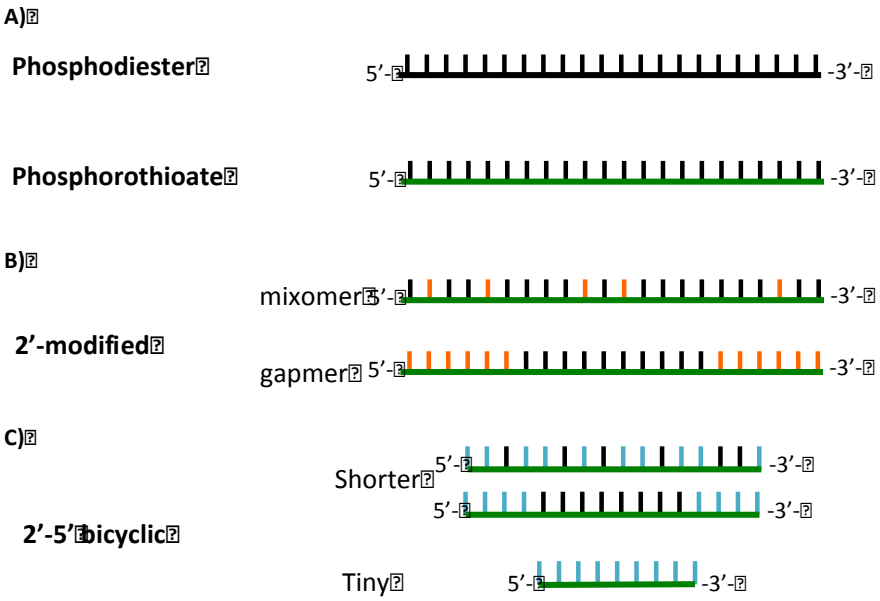

Figure 4: The three generations of antisense oligonucleotides. (A) Uniform modification of the ASO backbone from phosphorothioate (black line) to phosphodiester (green line; first generation) enabled better retention in circulation, significant loading in the liver and kidney and *in vivo* activity without delivery systems (Beltinger et al., 1995; Cossum et al., 1993; Crooke et al., 1996; Nolting et al., 1997) at a small cost of affinity for targets (Summerton & Weller, 1997). (B) Second generation, 2’-modified ASOs retain backbone modifications, are typically 20-30 nt long and are structured either with modifications (orange nucleosides) interspersed in the ASO (mixomers) or as a central 6-8 nucleoside stretch with no ribose modifications and flanked at the 5’ and 3’ ends with 2’- modified

nucleosides (gapmers). (C) Third generation 2'-5' chemistries (blue nucleosides) impart conformational rigidity that greatly enhances ASO affinity to their targets, increasing melting temperatures by as much as 4-8 ^{o}C per modified nucleoside (Kumar et al., 1998). Consequently, shorter ASO (e.g. 16-20 nt) can match or even outperform longer (20-30 nt) 2nd generation equivalents: bridged nucleosides force ASO not to tolerate base mismatches with their targets, thereby improving specificity (Valoczi et al., 2004). Short 100% modified octamers (tiny) may block entire miRNA classes with common seed sequences *en masse* (Obad et al., 2011).

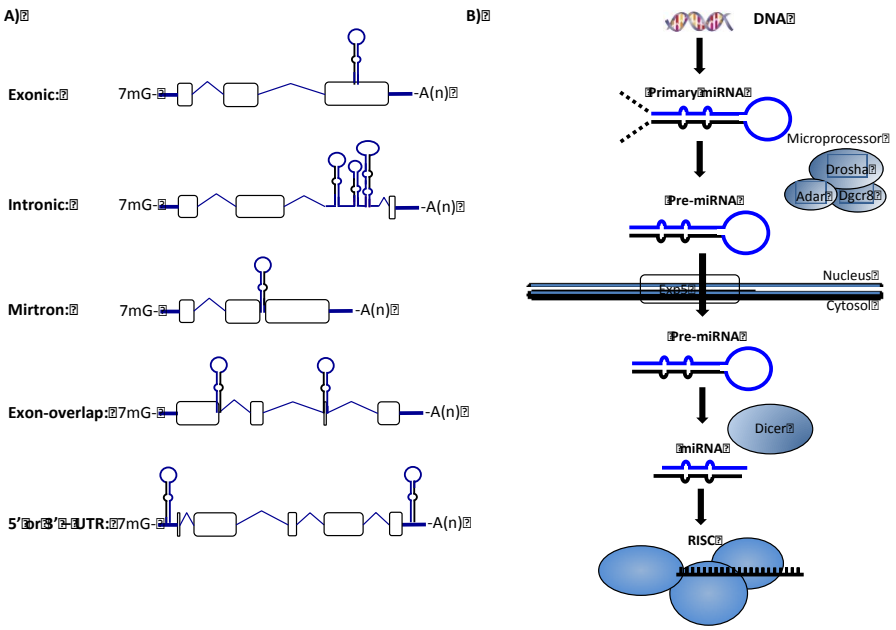

Figure 5: microRNA organization and maturation into bioactive RISC. (A) miRNA arise from exons (blue boxes) or introns (light blue angled lines). Some short introns can encode miRNA (mirtrons), whereas other miRNA precursors arise from exon-intron junctions, or

span entire short exons (exon-overlap). Yet others are processed out of 5’ and 3’ UTRs. (B) Endogenous RNAi mediator transcripts typically form stem-loop hairpin structures (primary miRNA) excised by the microprocessor complex in the nucleus into pre-miRNA and actively transported to the cytosol by Exportin 5. Here, the loop is endonucleotically removed by Dicer to form miRNA and then loaded onto AGO to form RISC; guide strand selection is driven by the orientation of the duplex loading onto AGO. Notable updates involve mitochondrial ncRNA-derived miRNA (mito-miRs) (Jagannathan et al., 2015; Ro et al., 2013), as well as AGO-directed small ncRNA (AGO-taxic ncRNA) of distinct genomic origin, but uniquely equipped to select AGO partners in RISC formation (Yamakawa et al., 2014). Small interfering RNA and miRNA replacement therapy can be achieved by gene therapy approaches exploiting any of these genomic organization structures or by providing exogenous, synthetic RNA (e.g. hairpins, duplexes) that engage with the latter processing steps of miRNA biogenesis. Adapted from (Moschos, 2013).

Table 1: Innate immune receptors for RNA and DNA oligonucleotide therapeutics.

Receptor	Location	Minimal motif	Oligonucleotide class affected	Evasion solution	Reference(s)
TLR1	Cell surface	Unknown	miRNA analogues	Unknown	He et al. 2013
TLR3	Cell surface	> 19 nt	siRNA, dsRNA	N6-methyladenosine, 2-thiouridine	Cho et al., 2009; Kleinman et al., 2008; Karikó et al., 2005
TLR7/8	Cell surface	UG	siRNA, miRNA, ASO	2' ribose modification, N6-methyladenosine, 2-thiouridine	Hornung et al., 2005; Judge et al., 2005; Fedorov et al. 2006; Jackson et al. 2006
TLR9	Cell surface	unmethylated CpG motifs	DNA ASO	CpG methylation	Krieg et al. 1995; Rutz et al 2004
RIG-I	Cytosolic	blunt duplex ends; 5' triphosphate	siRNA, dsRNA	3' dinucleotide overhangs, no 5' triphosphates	Marques et al., 2006; Kato et al., 2008
MDA5	Cytosolic	> 30 nt	dsRNA	short sequences	Kato et al., 2008
PKR	Cytosolic	> 30 nt	dsRNA	short sequences	Sledz et al., 2003
OAS1	Cytosolic	NNWW(N_9)WGN	siRNA, miRNA, RNA ASO	Design	Kodym et al., 2009
DAI	Cytosolic	> 30 nt	dsRNA	short sequences	Manche et al., 1992

Table 2: Types of negative controls for oligonucleotide therapeutics.

Control type*	Examples	Key features
Scrambled	5'-C**UG**GUUAGUGGCACUUCGAUU-3' to 5'-C**GU**GUUAGUGGCACUUCGAUU-3'	• impact increases with scrambling amount • may require >1 controls • avoid accidental host gene targeting • scramble 10-11 / central octamer to create slicer / RNase H control
Mismatch	5'-A**U**GGUUAGUGGCACUUCGAUU-3' to 5'-A**C**UGUUAGUGGCACUUCGAUU-3'	• seed region placement alters off targets** • slicer / RNase H active site inhibition • >1 controls also recommended
Reverse	5'-CUGGUUAGUGGCACUUCGAUU-3' to 5'-UUAGCUUCACGGUGAUUGGUC-3'	• check for unintended host hits • no complementarity to intended target
Validated bioactive oligo	Green fluorescent protein, luciferase, *Arabidopsis thaliana, C. elegans*	• target not normally found in host transcriptome • confirm lack of complementarity • confirm no seed sequence in pathway of interest
Commercial negative control	Research supplier 'universal' negative controls	• limited 'negative' validation • unknown modifications • unknown sequence

*: Must match design and modification criteria of active oligonucleotide.
**: Relevant to RNAi mediators only.

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